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Bacterial motility: a component in experimental *Pseudomonas aeruginosa* burn wound sepsis*

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Summary

Mutants which do not spread in soft agar were derived from a rat burn wound virulent strain of *Pseudomonas aeruginosa*. When inoculated on to rat burn wounds, these motility-altered strains had significantly reduced virulence. Control agar-spreading isolates that had undergone the same manipulations were found to have maintained virulence. Other than motility, no other parental characteristic was found to be altered. The non-spreading isolates were virulent when inoculated below the burn wound. It is concluded that motility is an important factor in experimental burn wound sepsis.

INTRODUCTION

THE characteristic factors which allow *Pseudomonas aeruginosa* to be a common and frequently successful opportunistic pathogen are not well defined. Investigations of possible pathogenic mechanisms have emphasized the reproduction of the clinical toxicity which frequently accompanies severe pseudomonas infection (Moncrief and Teplitz, 1964; Alexander, 1971). Several varieties of toxic extracellular products including toxins, haemolysins and proteolytic enzymes have been isolated (Muszynski, 1973; Liu, 1974 and Pavlovskis and Shackelford, 1974). The bioassay of the toxicity of these materials has generally been accomplished in mice. The roles of specific factors in the process of infection *per se* have only recently been addressed. Injection of pseudomonas strains with known *in vitro* capacity to produce specific toxins has implicated their importance *in vivo* (Stieritz and Holder, 1978; Wretlind and Kronevi, 1978). Such injections of organisms, however, would seem to avoid the requirement that a virulent strain colonize and invade the host.

We have examined bacterial motility as a requirement for virulence in a rat model of progressive infection following burn surface inoculation. The approach was to test for loss of virulence in non-motile isolates derived from a virulent, motile, parent strain.

MATERIALS AND METHODS†

Rat burn model

The Walker–Mason rat scald technique (Walker and Mason, 1968) was used. A 30 per cent full-thickness burn injury was inflicted on 350-g rats anaesthetized with sodium pentobarbital (25 mg/kg). Thermal injury was achieved after a 10-s exposure of the shaved dorsum to boiling water. No fluid resuscitation was administered.

Parental organism

Strain 59-12-4-4, a human blood isolate, was used. The rat virulence of this strain has been previously reported (Walker et al., 1964; McEuen et al., 1976).

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†In conducting the research described in this report the investigators adhered to the *Guide for Laboratory Animal Facilities and Care*, as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences National Research Council.

Mutagenesis and non-motile clone isolations

The parental strain was exposed for 1 h to nitrosoguanidine in a dose of 0·1 mg/ml in 0·1 M citrate buffer, pH 5·0, at 37 °C. Cells were then washed in Trypticase Soy Broth (TSB) and incubated for 12 h at 35 °C in a shaking water bath. The culture was then washed in TSB and serial 10-fold dilutions were prepared in TSB. The first 4 dilutions were plated (0·1 ml/plate) into 10 ml of one-third strength TSB containing 1 per cent $NaNO_3$ and 0·5 per cent agar. Fifty plates were prepared from each dilution and incubated at 35 °C for 24 h. Following incubation, plates were examined for non-'haloed' colonies. Sixteen non-spreading colonies were isolated. The isolated non-spreading clones were examined by hanging drop and incubated in TSB overnight. Following incubation, the isolates were examined for motility by hanging drop observation and by stabbing semi-solid medium (0·5 per cent agar).

Virulence testing

Parental, non-motile and control isolates were grown overnight in shaking TSB cultures. Inocula were diluted in TSB to 10^8 cfu/ml and 1 ml was spread over the scald wounds.

Non-motile isolates were also examined for virulence when inoculated below the burn wound. The inocula were 10^8 cfu (1 ml) given as four injections of 0·25 ml.

Growth comparisons

Strains were compared for relative growth rates in shaking TSB cultures. Growth was measured by plate counting as a function of culture age.

Protease production

Total protease activity was measured in culture filtrates of 24-h cultures grown in dialysed TSB. The growth medium was prepared by dialysing 3× concentrated broth against two volumes of deionized water. Filtrates were examined for total protease activity using commercially obtained (Sigma) hide powder azure as a substrate (Rinderknecht et al., 1968).

Electron microscopy

Morphological examinations were made on suspensions of agar-grown colonies dispersed in water and collected on Formvar grids. Grids were air-dried and shadowed with platinum.

Examination for metabolic alterations

Nitrosoguanidine mutagenesis has been reported to induce a high frequency of auxotrophic and cell membrane mutations (Holloway, 1975). Motility variants were examined for auxotrophy by measuring growth on minimal agar (Clowes and Hayes, 1968). Alterations in cell somatic antigens were examined serologically (Difco typing set). Strain metabolic activities were examined using the API taxonomic system. Changes in strain antibiograms were examined by the Kirby–Bauer technique (Bauer et al., 1966).

RESULTS

Strain isolations

Following TSB subculture, only five non-haloed isolates remained non-spreading in 0·5 per cent agar. The strains were designated M-5, M-6, M-9, M-10 and M-13. Spreading control isolates were designated W-1, W-2, W-3, W-4 and W-5. All strains were then stored at −80 °C in sterile milk. Further attempts to revert the agar non-spreading strains by static broth subculture were unsuccessful. Strain M-13 was found to be non-spreading in agar concentrations as low as 0·3 per cent, yet was motile by hanging drop in liquid culture. Organisms removed from non-spreading subsurface colonies of M-13 were motile when mounted in water. Strain M-6 was found to have a low (approximately 1 1000 cells) frequency of liquid-motile cells, but no agar-spreading clones could be demonstrated. *Fig.* 1 shows that upon electron microscopic observation, strains M-5, M-9 and M-10 were non-flagellated. Strains M-6, M-13 and the parent were monotrichous.

Rat burn wound virulence

Mortality data are presented in *Table I*. Animals whose burn surface was inoculated with non-spreading isolates had significantly increased survival when compared with those receiving control and parental strains ($P < 0{\cdot}01$). Chi-square analysis was used. Additionally, non-survivors in the non-spreading groups had an extended time to death when compared with control groups ($P < 0{\cdot}01$).

Injection of the non-spreading isolates into the burn resulted in increased mortality (33 50) when compared with the mortality observed with surface inoculation (19 50) ($P < 0{\cdot}01$). As a group, injected non-spreading isolates did not produce mortality equal to that observed with surface inoculation of control organisms ($P < 0{\cdot}01$).

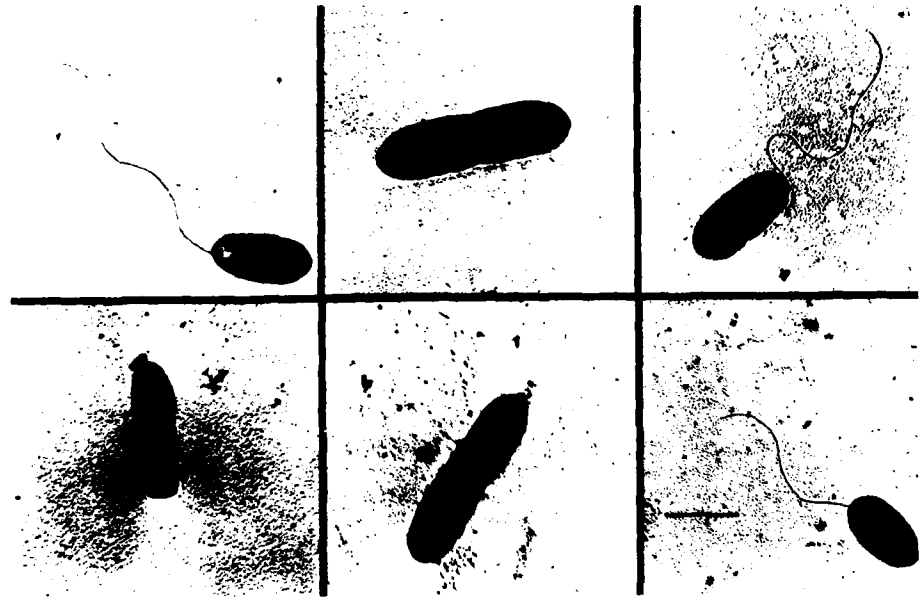

Fig. 1. Strain designations are from left to right: parent, M-5, M-6, M-9, M-10 and M-13. The bar represents 1 μm.

Table I. Rat burn wound virulence*

	Days post burn inoculation																					*Total dead*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21/28	
Non-motile																						
M-5										1		2					1	1				5/10
M-6												3		1	1	1	1					7/10
M-9																		1				1/10
M-10														1	1	1						3/10
M-13														1			1	1				3/10
Total																						19/50
Motile																						
W-1				1	1		1	1	1	1		3										9/10
W-2				1	1					1	1		1			2	2	1				10/10
W-3				2			1		1	1			1		1	1	1	1				10/10
W-4							1		1		3	3				1		1				10/10
W-5					1	1		1					1			2		3				9/10
Total																						48/50
Parent	1							4	2	3	1	2	5	1		1						20/20

*Mortality results following 10^8 cfu inoculation of burn wound surface of 30 per cent scalded 350-g rats.

Protease production

Data are presented in *Table II*. Analysis of variance showed no difference between the motile and non-motile groups but, within the non-motile group, protease production by strain M-9 was significantly lower than the mean of the remaining strains ($P < 0{\cdot}01$).

Growth, metabolic activity, serotype and antibiotic sensitivity comparisons

Neither non-spreading isolates nor control cultures had auxotrophic requirements; all strains grew on minimal agar. API codes were identical except for motility between parent and non-spreading strains. Regression analysis of the

Table II. Total protease activity*

Non-motile clones		*Motile clones*	
M-5	13·49 ± 0·47	W-1	13·85 ± 0·49
M-6	16·69 ± 1·48	W-2	12·94 ± 1·34
M-9	7·59 ± 1·94	W-3	14·09 ± 1·29
M-10	15·33 ± 2·45	W-4	13·58 ± 2·5
M-13	13·14 ± 1·22	W-5	17·02 ± 4·37
	Parent strain 12·96 ±0·31		

*Total protease activity in culture filtrates following 24-h growth. Data presented as means of duplicate samples run on triplicate cultures. Data are in trypsin activity equivalents (μg/ml) at pH 7·8 using hide powder azure substrate.

exponential growth curves showed no significant difference in growth rates, nor was any significant difference found between maximum stationary growth levels. Serotypes and antibiograms were unaltered.

DISCUSSION

Teplitz and his colleagues have described the histopathological similarity between experimental pseudomonas burn wound sepsis and the human disease (1964). In his elegant study, Teplitz followed the bacteriologic course from surface inoculation to death. The infection clearly progressed from colonization of the superficial eschar through massive accumulation of bacteria in non-viable tissue to invasion of viable hypodermal and adjacent tissue with subsequent haematogenous spread and death. The progressively invasive nature of the infection prompted us to ask what role motility might play in this disease.

In this report, selection for loss of colony spreading in soft agar resulted in diminished virulence. The differences in flagellar morphology and protease production indicate that these isolates are not strains of an expanding single mutant clone. No other known parental markers were changed. Loss of virulence was directly related to loss of agar spreading; unless one assumes that agar spreading is linked with other unknown virulence characteristics of the parent, or that mutagenesis resulted in loss of other unrelated virulence-linked markers which were randomly picked in the non-spreading strains and not in the motile controls. We feel that these alternatives are unreasonable. Bacterial motility is an important element in the pathogenesis of *P. aeruginosa* experimental burn wound sepsis. Additionally, these findings suggest bacterial motility as a future target in the development of antimicrobial and chemotherapeutic agents.

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Note

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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First Afro-Asian Conference on Burns

The first Afro-Asian Conference on Burns will be held in Bombay from 14 to 16 January 1981. It will be jointly sponsored by the Burns Association of India and the International Society for Burn Injuries.

The theme of the conference will be 'Prevention and management of burns in developing countries'. Presentations on these and all other aspects of burns will be welcome.

For details contact: Dr S. Agarwala, Organizing Secretary, c/o Burns Research Unit, Bai Jerbai Wadia Hospital for Children, Parel, Bombay 400 012, India.